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Trichoderma theobromicola and *T. paucisporum*: two new species isolated from cacao in South America

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ARTICLE INFO

Article history:
Received 1 September 2005
Received in revised form
14 November 2005
Accepted 16 January 2006
Published online 18 April 2006
Corresponding Editor:
David L. Hawksworth

Keywords:
Biocontrol
Hydrocrea
Moniliophthora roreri
Theobroma cacao

ABSTRACT

Trichoderma theobromicola and *T. paucisporum* spp. nov. are described. *Trichoderma theobromicola* was isolated as an endophyte from the trunk of a healthy cacao tree (*Theobroma cacao*, *Malvaceae*) in Amazonian Peru; it sporulates profusely on common mycological media. *Trichoderma paucisporum* is represented by two cultures that were obtained in Ecuador from cacao pods partially infected with frosty pod rot, *Moniliophthora roreri*; it sporulates sporadically and most cultures remain sterile on common media and autoclaved rice. It sporulates more reliably on synthetic low-nutrient agar (SNA) but produces few conidia. *Trichoderma theobromicola* was reintroduced into cacao seedlings through shoot inoculation and was recovered from stems but not from leaves, indicating that it is an endophytic species. Both produced a volatile/diffusable antibiotic that inhibited development of *M. roreri in vitro* and on-pod trials. Neither species demonstrated significant direct *in vitro* mycoparasitic activity against *M. roreri*.

Introduction

World cocoa (cacao, *Theobroma cacao*, *Malvaceae*) production is under severe threat from fungal pathogens wherever it is grown. In Latin America the most notable diseases are frosty pod rot, caused by *Moniliophthora roreri* which is a parasite endemic in the forests of northwest South America; witches' broom, caused by an Amazonian endemic pathogen *Crinipellis perniciosa* (Pound 1943; syn. *Moniliophthora fide* Aime & Phillips-Mora in press); and black pod, caused by various species of *Phytophthora*. Non-chemical control measures, such as farm sanitation, are labour intensive and often prohibitively expensive. Chemical control of these diseases is only cost-effective when the price of cocoa is high and the crop is under high disease pressure, and even then agents such as copper hydroxide may not save more than 20 % of a crop infected with frosty pod rot (Bateman *et al.* 2005). Biological agents that can be used in integrated strategies for the management of these diseases have been sought in the cacao-growing regions of Latin America.

Epiphytic and endophytic fungi are being pursued as potential biological control agents of the fungal diseases of

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doi:10.1016/j.mycres.2006.01.009

cacao. Endophyic fungi, in particular those that have coevolved with cacao or other *Theobroma* species, are being investigated for use as biocontrol agents within the framework of classical biocontrol (Evans *et al.* 2003). Searches for endophytic fungi of cacao and its relatives were conducted in the Amazon headwaters of Peru and specimens were isolated from wild trees (Evans *et al.* 2003; Holmes *et al.* 2004). This region is considered to be the centre of origin of cacao, due to the high diversity encountered (Bartley 2004) and to the discovery of germplasm resistant to *C. perniciosa* (Pound 1943). This pathogen is now on an invasive front in Latin America (Evans 2002). During one survey, a new *Trichoderma* species was isolated from within the stem of a mature, healthy cacao tree.

While searching for epiphytic fungi that can function as biocontrols in Ecuador, two non-sporulating cultures of a fungus were isolated from *M. roreri*-infected pods of *T. cacao* that had been placed in cacao leaf litter. These two cultures did not produce conidia reliably on common media except for synthetic low-nutrient agar (SNA), and then in low numbers. Both of these cultures had a strong odour of coconut, suggestive of many *Trichoderma* species.

The strong coconut odour produced by the Peruvian and Ecuadorian cultures is a distinctive feature of many members of the 'viride' clade (i.e.*Trichoderma* sect. *Trichoderma*, the 'rufa' clade of Chaverri & Samuels 2004), and all three of the cultures have been shown to produce nonanoic acid (M. Aneja and T. Gianfagna pers. comm.). Nonanoic acid is a short chain fatty acid known to inhibit spore germination and growth (Garrett & Robinson 1969).

This paper describes these two new species of *Trichoderma* from South America and their biocontrol potential.

Material and methods

Isolations

The Peruvian endophytic sample (DIS 85f) was obtained from live sapwood below intact bark of an old, multi-stemmed, asymptomatic cacao tree located in the floodwaters of the Rio Marañon (S04°24.17′, W73°28.57′) that was accessible only by canoe. The sampling procedure followed that described by Evans *et al.* (2003).

The Ecuadorian pod isolates (G.J.S. 01-13, 03-69) were obtained as follows. Preliminary studies (C.S. unpubl.) demonstrated that once cacao pods were on the ground they decomposed quicker than pods that continued to hang on trees. In a search for biocontrol agents against *Moniliophthora roreri* it was hypothesized that antagonists would colonise pods either soon after they fall, or that antagonists belonging to the normal surface microflora of the pod become more aggressive once the pod is on the ground. A study was set up in a 25-y-old plantation within the Pichilingue Research Station (Los Ríos, Ecuador). In the middle of the growing season when the pathogen was prevalent, pods that were up to one-third rotted by *M. roreri* were laid on a mulching layer below the trees, but not touching the soil. Three pods were sampled weekly over three weeks and isolations were taken from both the epi- and the mesoderm of the uninfected tissue of the pods. These samples were dilution-plated and all fungal colonies were isolated. Isolates G.J.S. 01-13 (Solis & Suarez 2.14) and G.J.S. 03-69 (Solis & Suarez 3.14) were recovered on two successive weeks (second and third week after the pods were placed on the ground) but they were not observed again.

Assessment of endophytic ability

The presumed endophytic isolate DIS 85f was tested for its ability to colonise cacao seedlings. The Ecuadorian isolates G.J.S. 01-13 and 03-69 were not tested because they were sterile or did not produce enough conidia for inoculation. In previous screening tests (Evans *et al.* 2003; Holmes *et al.* 2004), pre-germinated cacao beans were inoculated, but as these were not available, 8-week-old cacao seedlings (ex Costa Rica) were used. Six plants with meristematically active shoots or flushes were brush-inoculated with a spore suspension of *Trichoderma* (DIS 85f) containing 1×10^6 conidia in sterile distilled water (SDW) and 0.05 % Tween 20, using a fine camel-hair brush. The suspension was applied to both the unhardened stems and the expanding leaves of six plants and these were then transferred to a dew chamber (Mercia Scientific, Birmingham, UK) for 48 h, after which they were maintained in a quarantine CT room running at 22 °C with a 12 h light:12 h dark regime. Two further plants brushed with SDW only were included as controls. The inoculated, hardened flushes were harvested 10 d later and the stem cut into 1 cm sections and the leaves into 1 cm^2 pieces. These were sterilised in 1.5 % sodium hypochlorite for 10 min and vigorously washed three times in SDW, before being plated onto potato–carrot agar (PCA) and potato–dextrose agar (PDA). Plates were incubated at 23 °C and examined daily for the presence of *Trichoderma*. The experiment was repeated, but then the inoculated plants were left for one month before harvesting.

In vitro*: screening against* Moniliophthora roreri*: mycoparasitism*

All three isolates were screened for their mycoparasitic ability using a modification of a pre-colonised plate method (Foley & Deacon 1985; Krauss *et al.* 1998) as detailed in Evans *et al.* (2003). The target pathogen was inoculated near the edge of a 9 cm diam Petri plate containing PDA. When the plate was fully colonized a strip of inoculum of a *Trichoderma* isolate (0.5 × 2.5 cm), taken from the edge of an actively growing colony, was placed on the *Moniliophthora roreri* colony at the opposite edge from the *M. roreri* inoculum. The Petri plates were incubated for 24 h at 25 °C, after which time four evenly spaced, 5 mm diam plugs were taken from the distal edge of the *M. roreri* colony towards the *Trichoderma* inoculum. These plugs were plated onto PDA and incubated at 25 °C. Growth of the *M. roreri* or *Trichoderma* parasite from the plugs after 24 and 48 h provided a measure of the degree of colonisation of the pathogen by the *Trichoderma*. The experiment was performed with three isolates of *M. roreri*, *viz.* two isolated from pods of *T. gileri* in Ecuador (M.C.A. 2514, Phillips E42, DIS 116e) and one isolated from *T. cacao* in Costa Rica (M.C.A. 2504, Phillips C13). Two plates of each *Trichoderma*/*M. roreri* pair were prepared, and the experiment was repeated twice.

In vitro: *screening of an Ecuadorian isolate against* Moniliophthora roreri: *antibiosis*

Antibiotic production by culture G.J.S. 01-13 in confrontation with a local culture of *Moniliophthora roreri* was examined. In the first experiment, a 5 mm diam plug from actively growing cultures of G.J.S. 01-13 *M. roreri* were simlultaneously inoculated onto opposite sides of PDA in Petri plates. In the second experiment a Petri plate was inoculated with *M. roreri* and allowed to develop at 24 °C for 7 d. A 5 mm diam plug from the margin of an actively growing culture of G.J.S. 01-13 was placed in the Petri plate *ca* 1.5 cm distant from the growing edge of the *Moniliophthora* culture. The plates were incubated at 24 °C and observations were made for one month at intervals of two weeks. In both cases 10 Petri plates were used and each experiment was performed twice.

In vitro: *screening of the Peruvian isolate against* Moniliophthora roreri: *antibiosis*

The conidia of the *Trichoderma* isolate DIS 85f were harvested from two-week-old cultures grown on 20 % PDA at 25 °C and subsequently filtered through glass wool to remove mycelium. Three flasks containing 150 ml of 3 % Oxoid (Oxoid, Basingstoke, Hampshire, UK) malt extract broth (MEA) and three containing minimal salts broth (MIN; Srinivasan *et al.* 1992) were each inoculated with 1 ml of a 1×10^6 conidial suspension of the endophyte and incubated in an orbital incubator at 25 °C and 110 rev min^{-1} for 7 d. After 7 d growth the mycelium was removed by filtration and the filtrate sterilised by passing through a 0.22 μm membrane disposable filter unit (Millipore, Bellerica, Massachusetts, USA). The sterile culture filtrate was then stored at −20 °C before use. Before being incorporated into the medium the sterile filtrate was placed in a 90 °C water-bath for 2 h, after which it was added to an equal volume of the corresponding strengthened agar, 3 % MEA or 3 % MIN and poured into Petri plates. The plates were inoculated centrally with a 4 mm plug of *Moniliophthora roreri*, from the growing edge of a 7-d-old colony. Controls were prepared replacing the fungal filtrate with the corresponding uninoculated broth. Three replicate plates were used for each test and all plates were incubated at 25 °C. Inhibition of mycelial growth of *M. roreri* was recorded as the difference between mean radial growth in the presence and absence of the fungal filtrate.

In vitro: *screening against* Moniliophthora roreri: *pod assay*

Pods of *Theobroma cacao*, showing initial symptoms of natural infection with *Moniliophthora roreri*, were cut lengthwise and placed individually into plastic bags acting as moist chambers. A 7-d-old PDA culture of G.J.S. 01-13 was placed in each chamber, adjacent to one end of the pod. Development of the disease was monitored over 15 d. The assay was repeated twice.

Molecular characterisation

Fungal growth and DNA extraction were performed as reported previously (Dodd *et al.* 2002). The PCR for amplification of the internal transcribed spacer region (ITS1, 5.8 S and ITS2), a 0.65 kb section of translation elongation factor (*tef1*), 0.5 kb section calmodulin gene (*cal*), and 0.75 kb actin gene (*act*) was performed in a 50 μl reaction volume containing 5 μl of 10× PCR buffer (New England Biolabs, Ipswitch, MA), 200 μM dNTPSs (Promega, Fitchburg Center, Wisconsin, USA), 0.2 μM of each primer, 1.25 units of Taq Polymerase (New England Biolabs) and 10–50 ng of template DNA. The primers used for amplification of ITS were ITS1 and ITS4 (White *et al.* 1990). For *tef1* the primers were EF1-728F (Carbone & Kohn 1999) and TEF1 rev (Samuels, Dodd, Gams *et al.* 1987). For *cal* the primers were CAL-228F and CAL737R (Carbone & Kohn 1999). For *act* the primers were Tact1 (5'-TGGCACCACACCTTCTACAATGA) and Tact2 (5'-TCT CCTTCTGCATACGGTCGGA). The program used for amplification of all the genes consisted of 3 min initial denaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min and then a final 10 min extension. When amplification failed, the annealing temperature was lowered or the concentration of the DNA template in the PCR reaction was adjusted. The PCR products were purified using Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Sequence reactions were performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The products were analyzed directly on a 3100 DNA sequencer (Applied Biosystems). For each amplicon both strands were sequenced using the primers used to generate it. In the case of *act*, two additional internal primers Act500F (5'-ATTCCGTGCTCCTGAG) and Act511R (5'-CTCAGGAGCACG GAAT) designed during this study were used to complete the sequencing at 5' and 3' ends of the DNA fragment.

The sequences were assembled using Sequencher 3.1 (Gene Codes, Ann Arbor, Michigan), aligned using ClustalX 8.1 (Thompson *et al.* 1997) and then visually adjusted using MacClade 4.06 (Sinauer Associates, Sunderland, MA). Phylogenic trees were generated within PAUP 4.0b8 (Sinauer Associates, Sunderland, MA). We used *T. polysporum* Tr 46 and *T. minutisporum* DAOM 167069T (both belong to *Trichoderma* sect. *Pachybasium sensu* Bissett 1991, or Pachybasium 'B' *sensu* Kullnig-Gradinger *et al.* 2002) as outgroup for analysis of *tef1*, *act*, and *cal* individually and in combined sequence analysis. Gaps were treated as missing data. Phylogenic trees were generated using maximum parsimony and neighbour joining. Parsimony analysis was performed with a starting tree obtained via stepwise addition with random addition of 100 replications, tree-reconnection as the branch swapping algorithm, Mulltrees off. Stability of the clades was assessed with 500 bootstrap replications, using the settings described above. The data set derived from the combined three genes was also analyzed using neighbour-joining with the Kimura2 parameter model. Bootstrap values were calculated from 1000 replicates. The Genbank accession numbers for the sequences included in the analysis are listed in Table 1.

Phenotype characterisation

Growth rate and optimum temperature for growth were determined following the protocol of Samuels *et al.* (2002) on PDA

Table 1 – Isolates, their identification, origin and GenBank numbers

Species	Isolate	Origin	Gene			
			ITS	*tef1*	*cal*	act
Trichoderma theobromicola	DIS 85f	Peru	DQ109525	DQ109539	DQ122148	DQ111955
T. paucisporum	G.J.S. 01-13	Ecuador	DQ109526	DQ109540	DQ122149	DQ111956
T. paucisporum	G.J.S. 03-69	Ecuador	DQ109527	DQ109541	DQ122150	DQ111957
Trichoderma sp.	G.J.S. 00-14	Colombia	DQ109528	DQ109542	DQ122151	DQ111958
Trichoderma sp.	G.J.S. 00-15	Colombia	DQ109529	DQ109543	DQ122152	DQ111959
Hypocrea. flaviconidia	G.J.S. 99-49	Costa Rica	DQ023301	DQ020001		DQ111960
T. pubescens	DAOM 166162	USA: NC	DQ080316	AY750887	DQ122153	DQ111961
T. hamatum	DAOM 167057	Canada	Z48816	AF456911, AY750893	DQ122154	DQ111962
T. hamatum	G.J.S. 98-170	New Zealand	DQ109530	DQ109544	DQ122155	DQ111963
T. strigosum	DIS 173k	Brazil	DQ109531	DQ109545	DQ122156	DQ111964
T. viride VD	G.J.S. 89-142	USA: NC	DQ109532	AY376049	DQ122157	DQ111965
H. vinosa	G.J.S. 99-158	New Zealand	AY380904	AY376047	DQ122158	AY376680
T. viride / H. rufa	G.J.S. 99-83	Australia: Victoria	AF456921	AF348118	DQ122159	DQ111966
H. stilbohypoxyli	G.J.S. 96-32	Puerto Rico	AY380915	AY376062	DQ122160	AY376686
H. stilbohypoxyli	CBS 992-97	Puerto Rico	DQ109533	DQ109546	DQ122161	DQ111967
T. erinaceus	DIS 7	Peru	DQ109534	DQ109547	DQ122162	DQ111968
T. erinaceum	DAOM 230019	Thailand	DQ083009	AY750880	DQ122163	DQ111969
T. viride VB	Tr 22	USA:WA	DQ109535	AY937449	DQ122164	DQ111970
T. viride VB	Tr 21	USA: VA	AY380909	AY376054	DQ122165	AY376681
T. atroviride / H. atroviridis	CBS 142.95	Slovenia	AF456917	AF456891, AY376051	DQ122166	DQ111971
T. ovalisporum	DIS 172h	Brazil	AY380896	AY387660	DQ122167	AY376670
T. koningii / H. koningii	G.J.S. 96-120	The Netherlands	DQ109536	DQ109548	DQ122168	DQ111972
Trichoderma sp. TKON1	G.J.S. 96-120	Germany	DQ109537	DQ109549	DQ122169	DQ111973
T. asperellum	CBS 433.97	USA: MD	X93981	AF456907, AY376058	DQ122170	DQ111974
T. asperellum	G.J.S. 99-6	USA: GA	DQ109538	DQ109550	DQ122171	DQ111975
T. polysporum	Tr 46	USA: WI	DQ112552	AY750885	DQ122172	DQ111976
T. minutisporum/H. minutispora	DAOM 167069	Canada	DQ083015	AY750883	DQ122173	DQ111977

and SNA (Nirenberg 1976) without added filter paper. Colony radius was measured at 24, 48, and 72 h at 15, 20, 25, 30, and 35 °C. Each growth rate experiment was repeated three times and the results were averaged for each isolate. Additional characters, including the time of first appearance of green conidia, the presence of yellow pigmentation of young conidia, the occurrence of diffusing pigment in the agar, odour and colony appearance were also noted. Colony appearance was described from CMD (corn meal–dextrose agar, Difco, Michigan, USA, with 2 % w/v glucose) at 20 °C and PDA at 25 °C, including formation and shape of tufts or pustules.

Morphological observations were taken from cultures grown on CMD in 9 cm diam vented plastic Petri plates in an incubator at 20–21 °C, with 12 h fluorescent light and 12 h darkness ('light') within approximately one week. The following characters were measured: width of phialide base, phialide width at the widest point, phialide length, and length/width ratio (L/W), conidium length, width and length/width ratio (L/W), width of cell from which phialides arise (subtending cell), presence of chlamydospores, and chlamydospore width. Measurements of continuous characters were taken from images using the beta 4.0.2 version of Scion Image (Scion Corporation, Frederick, MD). Thirty units of each character were measured from water after initial wetting in 3 % potassium hydroxide for each collection. Measurements are reported as the extremes in brackets and the range calculated as the mean plus and minus the standard deviation.

The descriptive statistics and growth plots were calculated using Systat 10 (SPSS Inc., Chicago, IL).

Differential interference, phase contrast and epifluorescence microscopy were used. The fluorescent brightener calcofluor (Sigma Fluorescent Brightener 28 C.I. 40622 Calcofluor white M2R in 2 M phosphate buffer at pH 8) was used for epifluorescence microscopy.

Results

Endophytic inoculation

After 4–6 d DIS 85f grew from all the inoculated stem sections taken from the six test plants 10 d and one month after inoculation (Fig 1), but no growth was recorded on any of the inoculated leaf samples when the experiment was terminated three weeks later, nor from any of the control stems or leaves.

In vitro*: screening against* Moniliophthora roreri*: mycoparasitism*

The Peruvian endophyte, DIS 85f, was reisolated from all four plugs taken from M.C.A. 2514 (Ecuador, *Theobroma gileri*) within 24 h, but it showed modest (two plugs distal to the *Moniliophthora* inoculum) or no growth on DIS 116e (Ecuador, *T. gileri*) or M.C.A. 2504 (Costa Rica, *T. cacao*). In contrast, neither G.J.S. 01-13 nor G.J.S. 03-69 was reisolated from plates of M.C.A. 2514 after 48 h. The isolate G.J.S. 03-69 was not recovered from M.C.A. 2504 after 48 h and G.J.S. 01-13 was recovered only once from this *Moniliophthora* culture, and then from all

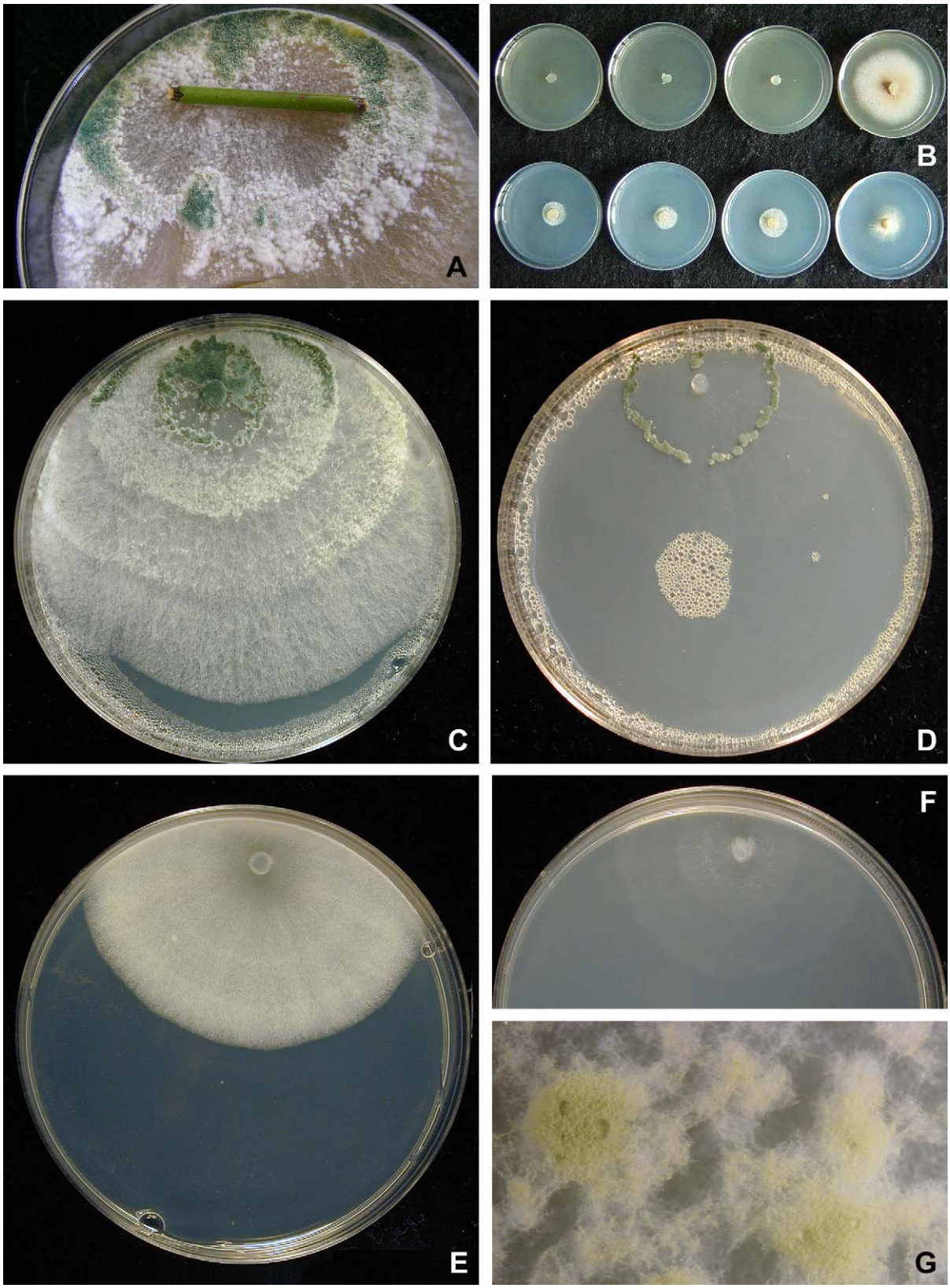

Fig 1 – (A) *Trichoderma theobromicola* reisolated from the upper portion of cacao stem 1 m after inoculation of cacao shoots with conidial suspension. (B) Soluble inhibitory metbolite production by *T. theobromicola* on MEA (top) and MIN (bottom). *Moniliopthora roreri* controls on the right. (C and D). *Trichoderma theobromicola* on PDA (C) and SNA (D) after 96 h in light. (E and F) *Trichoderma paucisporum* on PDA (E) and SNA (F) after 96 h in light. (G) *Trichoderma paucisporum* yellow conidia formed on half-strength PDA.

four plugs. Isolate G.J.S. 01-13 was reisolated from all four plugs of DIS 116e after 48 h and G.J.S. 03-69 was reisolated from all four plugs within 48 h in the second week but in the first week it was not reisolated within 48 h in one of the two plates and in the second plate it was only reisolated in three plugs distal to the *Moniliophthora* inoculum.

In vitro: *screening against* Moniliophthora roreri: *antibiosis*

When the *Trichoderma* G.J.S. 01-13 and the local isolate of *Moniliophthora roreri* were inoculated simultaneously, the *Moniliophthora* failed to grow after one month. When the *Trichoderma* was inoculated onto a 7-d-old plate that had been preinoculated with *M. roreri*, *M. roreri* did not grow further and it stopped sporulating. In some of the plates, the *M. roreri* culture turned into a thick yellowish, sterile stroma. In the control plates, *M. roreri* continued to grow and sporulate.

In vitro: *screening of the Peruvian isolate against* Moniliophthora roreri: *soluble inhibitory metabolite production*

As can be seen from Table 2 and Fig 2, DIS 85f completely inhibited radial growth of *Moniliophthora roreri* after 7 d on MEA and on MIN medium inhibition of radial growth of *M. roreri* was approximately 45 %.

In vitro: *screening against* Moniliophthora roreri: *pod assay*

Using isolate G.J.S. 01-13, after 4 d there was no visible development of *Moniliophthora roreri* at the end of the pod adjacent to the Petri plate with the *Trichoderma* culture. After a further 2–3 d, *M. roreri* on the rest of the pod was transformed to a thick, orange, sterile, pseudostromatic layer, similar to what was seen on Petri plates. On control pods that were not exposed to the *Trichoderma*, the *M. roreri* covered the pods and typically produced copious amounts of spores.

Phylogeny

Initially parsimony analysis of ITS1 and 2 and 5.8 S sequence of the endophytic *Trichoderma* strain DIS 85f and the two Ecuadorian pod cultures (G.J.S. 01-13, G.J.S. 03-69), along with sequences of identified species of *Trichoderma/Hypocrea* from three sections, *viz. Trichoderma* (incluging Pachybasium 'A' of Kullnig-Gradinger *et al.* 2002), Pachybasium 'B' (Kullnig-Gradinger *et al.* 2002), and *Longibrachiatum*, was performed. The phylogenic tree (not shown) positioned the three unknown isolates within *Trichoderma* section *Trichoderma*. However, ITS provides poor resolution for closely related species (Druzhinina & Kubicek 2005) and thus sections of three additional genes, *viz.* translation–elongation factor 1-α (*tef*), actin (*act*) and calmodulin (*cal*) were sequenced for these isolates as well as for representatives of known species of *Trichoderma* sect. *Trichoderma*. As outgroup we chose two species in Pachybasium 'B' (Kindermann *et al.* 1998), which is a sister group to sect. *Trichoderma*: *T. polysporum* Tr 46 and *T. minutisporum* DAOM 167069.

The combined sequence data of the three genes (Fig 2) included 1852 characters of which 1255 were constant, 409 were parsimony informative (22 %) and 188 were parsimony uninformative. The phylogenetic tree (Fig 2) based on parsimony analysis of the combined sequences revealed that when *T. polysporum* and *T. minutisporum* were used as outgroup taxa, with the exception of *T. asperellum*, the taxa in sect. *Trichoderma* split into two major clades, *viz.* the 'viride' clade and the 'hamatum' clade. The 'viride' clade included *T. viride* VD and VB, *T. erinaceus*, *T. koningii*, *T. ovalisporum*, *T. strigosum*, *Hypocrea stilbohypoxyli*, *T. atroviride* and the undescribed species *T.* cf. *koningii* Tkon21 (Holmes *et al.* 2004). The 'hamatum' clade included the three unknown isolates plus two *T. hamatum*, one *T. pubescens*, and two of an undescribed *Trichoderma* species from Colombia. Within the later clade the three unknown isolates formed a distinct clade in which the isolates G.J.S. 01-13 and G.J.S. 03-69 formed a highly supported (100 % BS) subclade for which the isolate DIS 85f formed a highly supported (100 % BS) basal branch. It is important to note that the clade of the three unknowns existed in the trees inferred from the analysis of sequences of individual genes *tef1*, *act* and *cal* with BS above 90 %. The individual gene parsimony trees are not shown here but the bootstrap values for the branches that occurred in all the three trees are indicated with a * under the branches in Fig 2. The entire 'hamatum' clade, including the three unknowns, is moderately to weakly supported with 63 % BS. *Trichoderma asperellum* was basal in sect. *Trichoderma* to the 'viride' and 'hamatum' clades. The neighbour-joining tree for the combined data set (tree not shown) had similar topology to the parsimony tree with exception of the *T. asperellum* clade, which was positioned within the 'hamatum' clade. As in the parsimony tree, the clade of the three unknown isolates was highly supported (Fig 2).

The isolates G.J.S. 03-69 and G.J.S. 01-13 have identical ITS, *tef*, *act*, and *cal* sequences and thus are considered to represent one species and possibly a single clone. DIS 85f showed strong phylogenic relationship with these two strains based on analyses of *tef1*, *cal*, *act* and the combined data set of the three gene sequences. However, the branch length between DIS 85f and G.J.S. 01-13/G.J.S. 03-69 is longer than those between any two isolates of the same species in sect. *Trichoderma*, e.g. *T. hamatum*, *T. viride* VD or VB or *T. asperellum* and thus we consider isolate DIS 85f to represent a distinct taxon and that additional sampling could reveal that these two taxa are not as closely related as they appear.

Table 2 – Percentage inhibition radial growth of *Moniliophthora roreri* after 7 d on malt extract broth (MEA) and minimal salts broth (MIN) containing culture filtrates of *Trichoderma theobromicola*

Rep.	Percentage inhibition of radial growth on MEA and MIN media	
	MEA	MIN
1	100.0	44.93
2	100.0	44.93
3	100.0	41.75

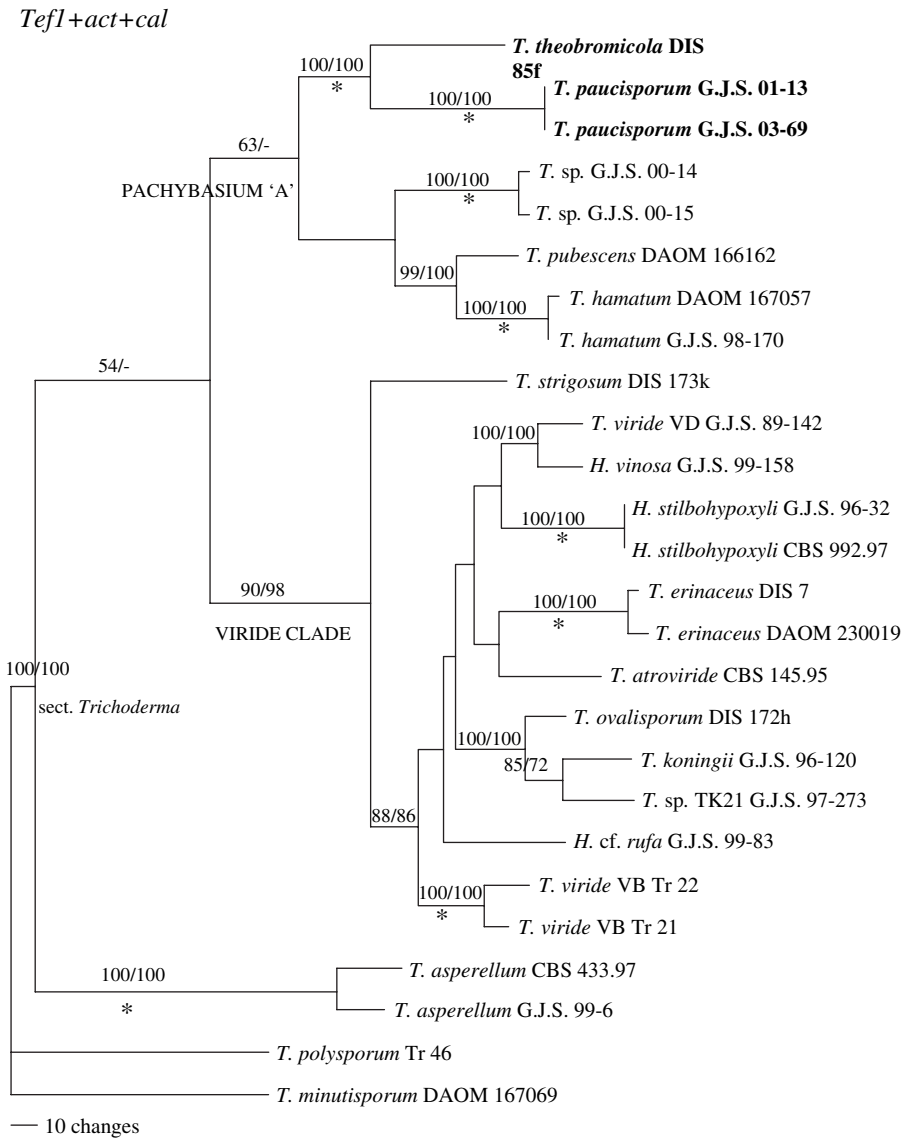

Fig 2 – The single most parsimonious tree obtained by phylogenetic analysis of combined data sequence from *tef1*, *cal* and *act*. Steps: 1217; consistency index: 0.63; retention index: 0.68. Outgroup taxa: *T. polysporum* and *T minutisporum*. The two numbers above branches are bootstrap values greater than 50 % for the parsimony tree from 500 replicates and the neighbour-joining tree from 1000 replicates, respectively. (*) Below branches refers to bootstrap value of above 90 % for the branch in all the three trees inferred from parsimony analysis of sequence data of *tef1*, *act* and *cal* individually.

Phenotype

Despite the apparently close similarity of the three strains in their DNA sequences, there are striking phenotypic differences. Most obviously, the Peruvian endophytic strain DIS 85f sporulates profusely on common media. The two Ecuadorian isolates only produced conidia consistently on SNA incubated at 20 °C under 12 h cool white fluorescent light and 12 h darkness. These isolates did not produce conidia, or only sporadically produced conidia, on various common mycological media, including full and half-strength PDA, CMA with and without 2 % glucose, and oatmeal agar (Gams *et al.* 1987) when incubated in darkness or under cool white fluorescent light or near UV light. Cultures incubated on autoclaved (15 psi for 20 min) rice incubated at 25 °C under cool white fluorescent light did not produce conidia. Although both G.J.S. 01-13 and G.J.S. 03-69 produced conidia on SNA as described above, there were far fewer conidia on Petri plates with the latter. In these two strains on SNA within 10 d inconspicuous, scattered, minute pustules, <1 mm diam, formed around the margin of the colony (Fig 4) or conidiophores arose singly from aerial hyphae (Fig 4); conidia remained hyaline for a long time before becoming green or they never turned green. Conidiophores in the pustules were profusely branched and typical of members of the 'viride' clade. Occasionally on CMD, PDA and SNA a few conidia of G.J.S. 01-13 formed from monophialidic, acremonium-like or verticillium-like conidiophores (Fig 4). In one trial

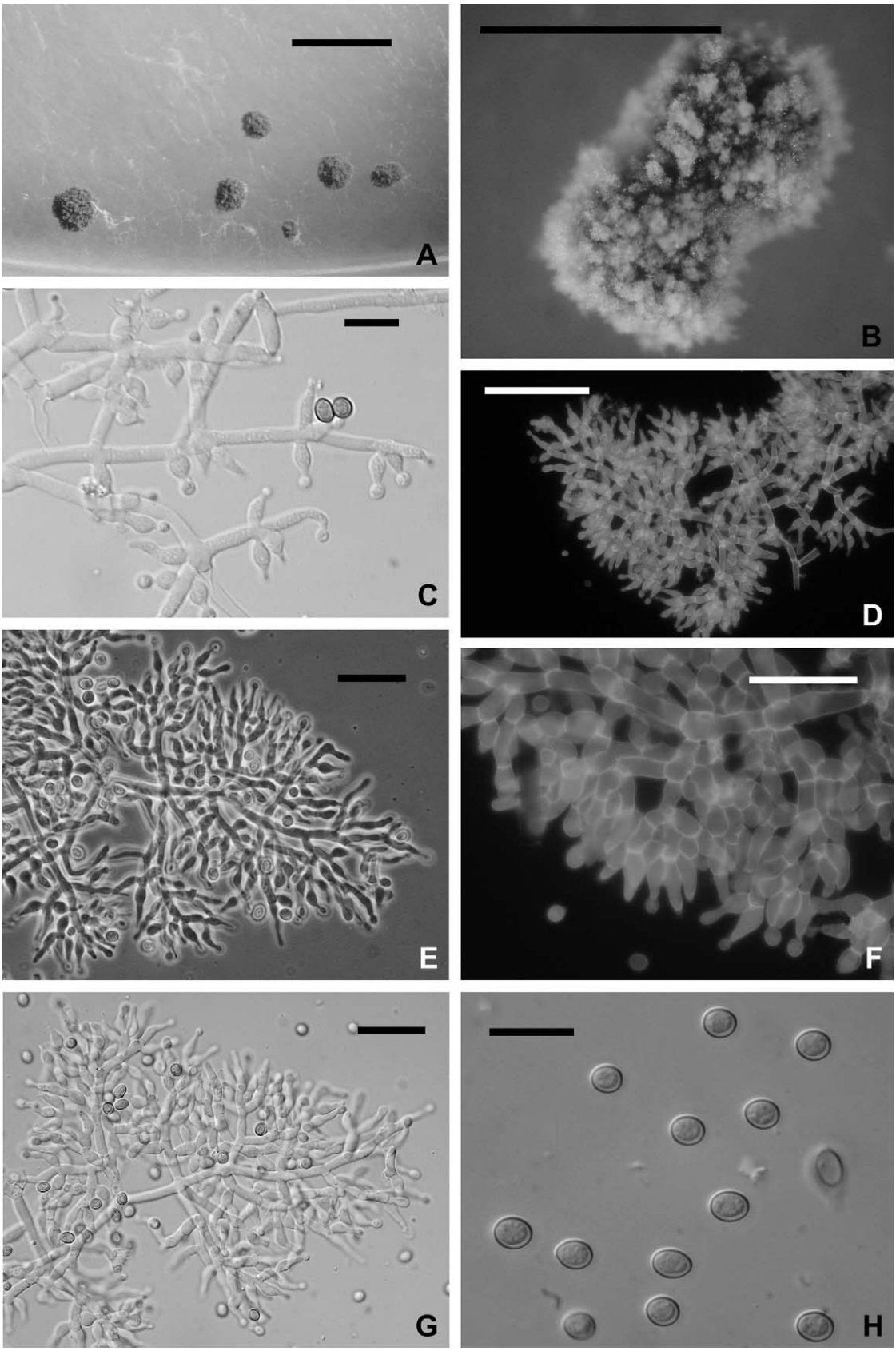

Fig 3 – *Trichoderma theobromicola*. (A and B) Pustules formed on CMD. Note the papillate aspect in B. (C–H) Conidiophores. (H) Conidia. All from DIS 85f on CMD. (C, G) DIC. (D, E) phase contrast microscopy. (F) Fluorescence microscopy. Scale bars: (A) 1 mm, (B) 0.5 mm; (C–G) 20 μm; (H) 10 μm.

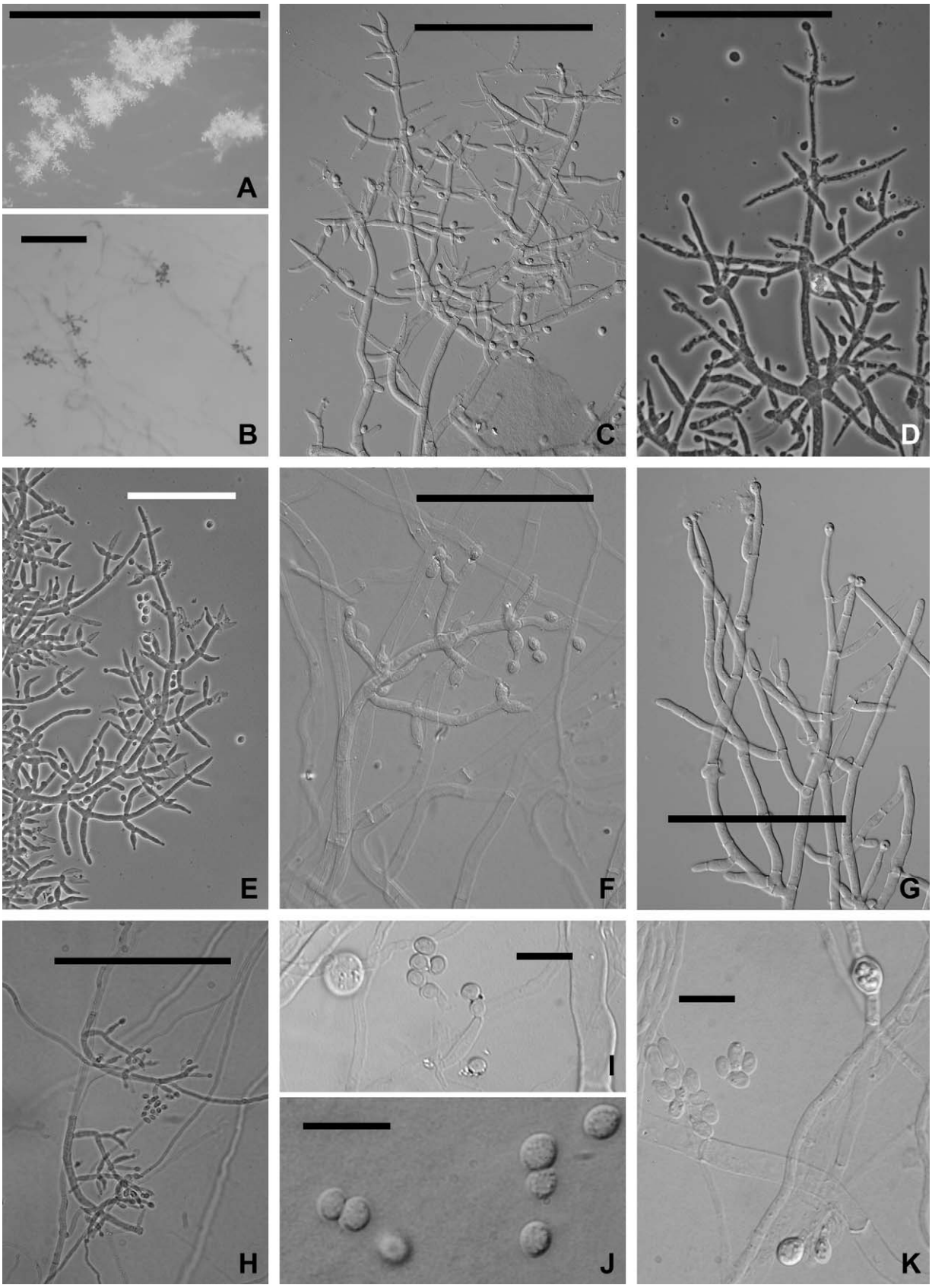

Fig 4 – *Trichoderma paucisporum*. (A–I) Conidiophores. Monophialidic conidiophores in (I). (J) Conidia. (K) Chlamydospores. All on SNA except Fig 4D (CMD). (A–B) stereo microscope, (C, F, H, I–K) DIC, (D, E) phase contrast microscopy, (H) bright field microscopy. Scale bars: A = 0.5 mm, B = 250 μm; C–H = 20 μm; I–K = 10 μm.

G.J.S. 01-13 produced conidia in three of five Petri plates containing half-strength PDA. The conidia were produced profusely in a continuous ring behind the edge of the Petri plate and conidia were yellow or slowly turned yellow green (Fig 3). The culture G.J.S. 03-69 remained sterile on CMD and PDA.

DIS 85f is readily recognizable as a *Trichoderma* species because of the copiously produced green conidia. Conidia are formed in compact, papillate pustules on CMD (Fig 3) or in dense, thick lawns on PDA (Fig 1A, B). Conidiophores (Fig 3C–G) of DIS 85f are richly and regularly branched with two branches tending to arise at each node; the internodal distances are short and the branch length increases with distance from the tip. In the pustules they are densely aggregated, giving the pustule a papillate aspect (Fig 3B). Phialides are straight to slightly sinuous, relatively long and slender, with L/W 1.8–3.4; they tend to be held in compressed, or gliocladium-like heads at the tips of branches of the conidiophores (Fig 3F). Conidia (Fig 3H) are broadly ellipsoidal to subglobose and unicellular.

The most distinctive aspect of this *Trichoderma* is the combined attributes of the very compact, papillate pustules, the conidiophore branching and arrangement of the relatively long and slender phialides. The morphology shown in Fig 3F is reflective of its position in the 'hamatum' clade.

All three cultures produced a strongly aromatic odour of coconut and G.J.S. 01-13 and G.J.S. 03-69 produced a pale to more conspicuous yellow pigment on PDA that diffused through the agar. In the later two isolates, the coconut-like odour has diminished over time.

Taxonomy

Because the two Ecuadorian isolates, G.J.S. 01-13 and G.J.S. 03-69, are identical in all four genes, *viz.* ITS, *cal*, *act*, and *tef*, as well as in their respective phenotypes, they represent a single species and most likely a single clone. The endophytic DIS 85f differs conspicuously from these two isolates in phenotype despite their apparent close relationship. The differences in biology and phenotype, combined with differences in their DNA sequences lead us to recognize two cacao-related species, neither of which can be accommodated in any of the known species of *Trichoderma*.

Trichoderma theobromicola Samuels & H.C. Evans, **sp. nov.** (Figs 1C,D, and 3)

Etym.: '*theobromicola*' refers to a possibly commensal relationship of this species with *Theobroma cacao* trees.

Conidiophora in pustulis compactis aggregata, saepe et aequaliter ramosa. Phialides cylindricae vel lageniformes, (5–)5.5–9.5(–12.5) × 2.5–3.5(–4) μm. Conidia late ellipsoidea ad ovoidea, viridia, glabra, 3.5–4.5 × 3–3.5(–4) μm, ratio longitudinis latitudine (1–)1.1–1.3(–1.4). Incrementum radiale in agaro dicto 'PDA' post 96 h ad 25 °C 50–65 mm.

Typus: **Peru**: *Loreto Dept.*: Río Marañon, Betsaida, forest edge along river, isolated from trunk of living tree of *Theobroma cacao*, May 1999, *H.C. Evans & D.H. Djeddour DIS 85f* (BPI 871726 — holotypus; ex-type cultures IMI 393419, CBS 119120, ATCC MYA-3640).

Optimum temperature for growth on PDA and SNA 25 °C; colony radius after 96 h in darkness on PDA 50–65 mm, on SNA 20–30 mm. Colonies on PDA after 96 h in darkness or in light producing barraging rings of aerial mycelium, conidia forming profusely in dense, flat areas around the centre of the colony; on SNA conidia forming in a single ring of confluent, pulvinate pustules (Fig 1D).

Colonies grown on CMD *ca* one week >90 mm diam, no diffusing pigment, a strong, sweet coconut-like odour. Conidia forming around the periphery of the colony in conspicuous, dark green, hemispherical pustules up to 1 mm diam; pustules lacking hairs, very dense, the surface ± papillate from tightly bound conidiophores, and conidia appearing moist; conidia forming sparingly apart from the pustules. *Conidiophore* with a discernable central axis and profuse 1° branches; 2–4 1° branches arising at or near 90° with respect to the main axis at a single node, distance between nodes short, 1° branches progressively longer with distance from the tip of the main axis, producing 2° branches; 2° branches producing phialides at or near the tip singly or in appressed, penicillate whorls of 2–4. Phialides straight or, less frequently sinuous or curved, nearly cylindrical to lageniform and somewhat swollen in the middle, not sharply constricted at the tip, (5.–)5.5–9.5 (–12.5) μm long, 2.5–3.5(–4) μm wide at the widest point, L/B = (1–)2–3.5(–4.5), (1.–)1.5–2.5 μm at the base, arising from a cell 2.5–3.0 μm wide. Intercalary phialides sometimes seen. *Conidia* broadly ellipsoidal to subglobose, 3.5–4.5 × 3–3.5 (–4) μm, L/B = (1–)1.1–1.3(–1.4), smooth. *Chlamydospores* not observed.

Known distribution: Known only from the original isolation.

Trichoderma paucisporum Samuels, C. Suarez & Solis, **sp. nov.** (Figs 1E–G, and 4)

Etym.: '*paucisporum*' refers to the sparse conidial production by this species.

Conidiophora pauca, inconspicua, mononemata vel ramosa. Phialides cylindricae, (6.2–)6.7–12.7(–21.5) μm × (2–)2.5–3.5 μm. Conidia subglobosa, viridia, glabra (3–)3.5–4.5(–5) × 2.5–3.5 μm, ratio longitudinis latitudine (1.0–)1.2–1.4. Incrementum radiale in agaro dicto 'PDA' post 96 h ad 25 °C circa 25 mm.

Typus: **Ecuador**: *Los Rios Prov.*: vic. Quevedo, Pichilingue, Estacion Experimental de Pichilingue, 1999, isolated from pods of *Theobroma cacao* partially infected with *Moniliophthora roreri* lying on leaf litter, *C. Suarez & K. Solis 2.14* (BPI 870953—holotypus; ex-type cultures G.J.S. 01-13, CBS 118645, ATCC MYA-3641). A second isolation, same location, *K. Solis 3.14* (culture G.J.S. 03-69, CBS 118645, ATCC MYA-3642).

Optimum temperature for growth on PDA and SNA 25 °C, colony radius after 96 h in darkness on PDA 24–26 mm, at 20 °C 18–21 mm, at 30 °C 20–25 mm. On SNA at 25 °C 22–25 mm, at 20 °C 16–20, at 30 °C *ca* 20 mm. Colonies on PDA after 96 h in darkness or under 'light' off white to ivory, with scant, felty aerial mycelium, pale yellow diffusing pigment, a coconut odour or no distinctive odour, sterile. On half strength PDA sometimes producing conidia in broad, felty bands; conidia remaining yellow or becoming yellow–green. Colonies on SNA nearly invisible with scant aerial mycelium in the centre of the colony, no diffusing pigment, sometimes a strong odour of coconut detected, producing conidia in minute pustules around the margin; conidia remaining hyaline or slowly becoming green. Colony radius <5 mm at 35 °C on PDA, not growing at 35 °C on SNA. On SNA within 10 d conidia barely visible, few on poorly formed conidiophores around the periphery of the colony or arising from conidiophores in

minute pustules <1 mm diam; on CMD few inconspicuous conidia formed after two week on acremonium-like, monophialidic conidiophores scattered throughout the colony. *Conidiophores* on SNA macronematous, monophialidic and acremonium-like or variously branched and tending to have a well-developed main axis with lateral branches tending to be paired or arise in divergent whorls of up to three, often solitary phialides arising directly from the main axis; 1° branches producing phialides directly singly and in whorls of three, terminating in 1–3 phialides in a whorl. *Phialides* (SNA) lageniform and slightly swollen in the middle or tapering uniformly from base to tip, (6–)6.5–12.5(–21.5) μm long, (2–)2.5–3.5 μm at the widest point, (1.5–)2–2.5 μm at the base, arising from a cell (2–)2.5–3 μm wide. *Conidia* (SNA) ellipsoidal to broadly ellipsoidal (3–)3.5–4.5(–5) × (2.5–)2.5–3.5 μm, smooth, green, L/B = (1.0–)1.2–1.4. Chlamydospores sometimes produced abundantly on SNA, CMD and half strength PDA; on SNA terminal on hyphae or less frequently intercalary in hyphae, becoming thick-walled, subglobose to ellipsoiodal, (6.5–)7–9.5(–12) × (4.5–)5.5–7.5(–9) μm.

Known distribution: Known only from the type locality.

Discussion

Trichoderma paucisporum and *T. theobromicola* are phenotypically distinct but phylogenetically closely related members of the 'hamatum' clade of *Trichoderma* (Fig 2; i.e. *Trichoderma* sect. *Pachybasium* 'A' in the sense of Kullnig-Gradinger *et al.* 2002 and Jaklitsch *et al.* in press). The 'hamatum' clade is a moderately well-supported sister clade to the 'viride' clade (i.e. *T.* sect. *Trichoderma*, the 'rufa' clade in Chaverri & Samuels 2004; Jaklitsch *et al.* in press), the core of *Trichoderma* because it includes the type species of the genus, *T. viride*, in addition to *T. koningii* and *T. atroviride*.

The new species are unusual in that none of the previously known members of the 'hamatum' clade produce a sweet, coconut-like odour whereas its presence is common in the 'viride' clade especially so in *T. atroviride*. With the exception of a new species of *Hypocrea* (Jaklitsch *et al.* in press) that is intermediate between the 'viride' and the *T. polysporum* clades (i.e. 'Pachybasium B,' Kullnig-Gradinger *et al.* 2002), we have not detected this odour outside of the larger sect. *Trichoderma*. Interestingly, this odour is characteristic of 6-pentyl-alpha-pyrone (6PAP), a compound that has been reported from the unrelated *T. harzianum* (Rocha-Valadez *et al.* 2005); however, we have never detected the odour in cultures of *T. harzianum*.

All three of the cultures have been shown to produce both nonanoic acid and 6PAP (M. Aneja and T. Gianfagna pers. comm.), compounds known to inhibit germination of spores of plant pathogenic fungi (Smith & Grula 1982; Wainwright 1982; Stefanova *et al.* 1999).

The ability to reintroduce *T. theobromicola* into cacao seedlings supports previous evidence (Evans *et al.* 2003) that *Trichoderma* spp. isolated from the stems of wild *Theobroma* trees can invade and endophytically colonise unhardened cacao stems or flushes but not developing leaves.

Although we were not convincingly able to demonstrate mycoparasitism of either *T. paucisporum* or *T. theobromicola*, *in vitro* and on-pod trials demonstrate that both have an antibiotic effect against *Moniliophthora roreri*. The discovery that both species produce nonanoic acid *in vitro* also suggests that this metabolite could be produced *in planta*. As regards *T. theobromicola*, which was isolated as an endophyte, the possibility that it could contribute to induced host resistance to disease, suggests that host and endophyte are in a coevolved symbiotic relationship. That either or both of these species can induce resistance, or protect pods from infection, offers a strong possibility that they can be exploited for biological control of the highly destructive and invasive cacao pathogen *M. roreri*.

Acknowledgments

Orlando Petrini corrected the Latin descriptions. Priscila Chaverri (Howard University) contributed significantly to the phylogenetic analysis. Thomas Gianfagna and Madhu Aneja shared with us their results of nonanoic production. Cathie Aime (BPI) provided cultures of *Moniliophthora roreri*. Thanks to Jayne Crozier (CABI-Bioscience) for technical assistance in carrying out the soluble metabolite studies and to Dylan Irion (BPI) and Lutorri Ashley (BPI) for additional technical assistance.

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